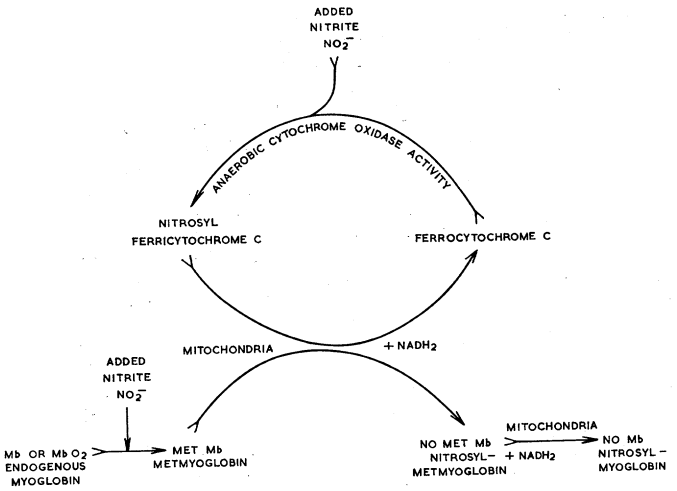


*Fig. 8. Suggested mechanism for formation of nitric oxide myoglobin during curing.*

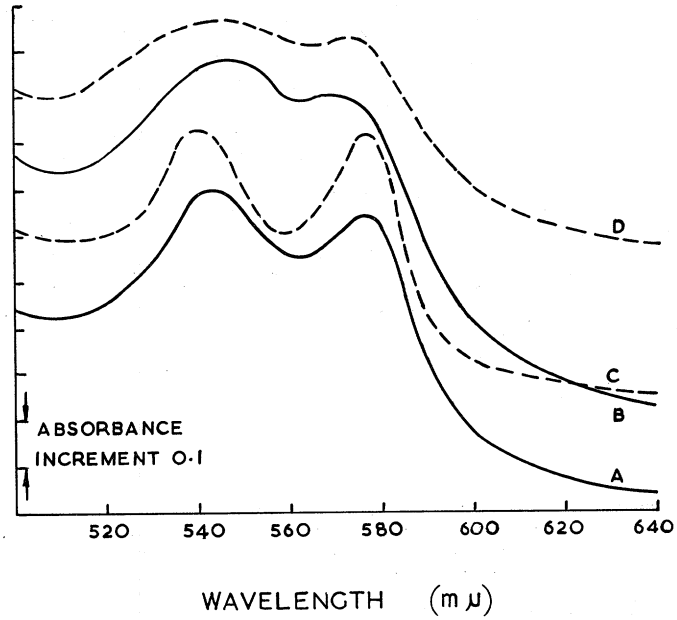


*Fig. 9. Visible spectra of products of anaerobic incubation of brine organism inoculum (A) and pig muscle mince (B) with nitrite in appropriate nutrient medium containing defibrinated horse blood, with reference spectra of hemoglobin in defibrinated horse blood in the oxy- (C) and nitric oxide- (D) forms.*

bility of microbiological reduction of these salts to nitric oxide by curing brine organisms must be considered. To investigate this point, three long-established Wiltshire curing brines were examined. The brine used most extensively had been in use for approximately 9 years; it was of pH 6.9 and contained 23.8% NaCl, 0.28% $NaNO_2$, and 0.94% $KNO_3$. Aerobic plate counts yielded an average of $15 \times 10^6$ organisms per ml and anaerobic $7.5 \times 10^6$.

Differential absorption manometry (Walters and Taylor, 1963) showed no detectable formation of a nitric oxide gas fraction after anaerobic incubation of this brine for 90 min at 37°C with additional nitrite to a total of 4300 ppm; attempts to detect nitric oxide in the absorption solutions by spectrophotometry (Walters and Taylor, 1964b) were also negative. Application of a modification of the Hornsey (1956) technique of extraction into aqueous acetone to the products of overnight incubation of the same brine at 25°C under anaerobic conditions in the presence of reduced myoglobin gave no indication of the formation of any nitric oxide pigment.

Finally inoculations of the brine (and of two other old-established Wiltshire curing brines) were made into a medium designed to promote vigorous growth of the organisms present and the inoculated media were incubated anaerobically for 14 days at 25°C. In view of the known sensitivity of myoglobin to autoxidation under anaerobic conditions, defibrinated horse blood was added to introduce haemoglobin as a more sensitive nitric oxide acceptor. The medium contained initially 200 ppm of nitrite; after 14 days' incubation all three brines had effected virtually complete utilization of the available nitrite, residual amounts being 1 ppm or less. In spite of the evident nitrite-reducing action of the brine organisms, there was no spectrophotometric indication of the formation of any nitric oxide pigment complex.

Fig. 9 compares the visible spectrum of the incubation products from a brine inoculation with that of the products from a similar incubation in which the brine inoculum was replaced by a fresh mince of pig quadriceps femoris muscle, chloromycetin being included to suppress bacterial growth. Reference spectra of the haemoglobin in the defibrinated horse blood in the oxy- and nitric oxide-forms are included and the difference in behavior between the brine and the muscle incubation is evident.

The difference Soret spectrum of the products of incubation of the brine inoculation with reference to those of the incubation containing muscle mince corresponds closely with that of oxyhaemoglobin with reference to nitric oxide haemoglobin (Fig. 10). Nitric oxide displaces oxygen directly from oxyhaemoglobin to form nitric oxide haemo-

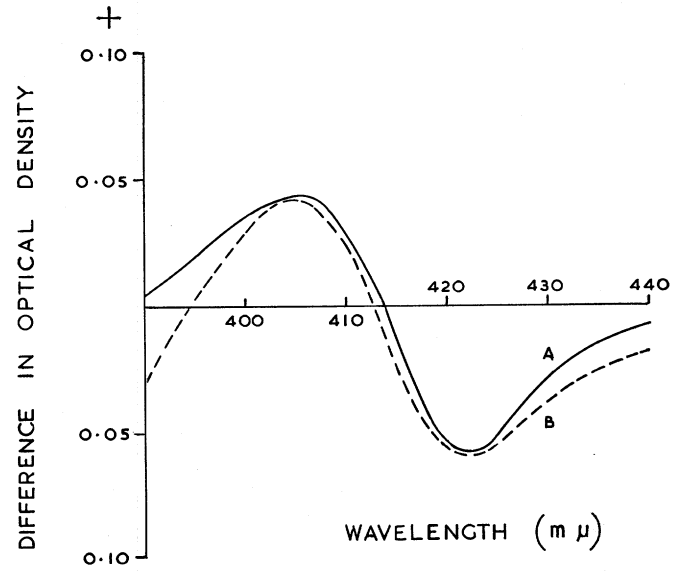


*Fig. 10. Difference Soret spectrum of diluted products of anaerobic incubation of brine organism inoculum with nitrite in appropriate nutrient medium containing defibrinated horse blood with reference to that of a similar incubation of pig muscle mince (A) and of oxyhemoglobin with reference to nitric oxide hemoglobin (B).*

globin, so that no difficulty should have been encountered in detecting any formation of nitric oxide by this means.

Baalsrud and Baalsrud (1954) and Najjar and Allen (1954) obtained nitric oxide as a product of metabolism using whole bacteria and aqueous extracts respectively. However Verhoeven (1956) concluded that the production of nitric oxide by bacterial action is a metabolic aberration, taking place only under the special conditions of a disturbed enzyme system. According to Verhoeven, nitrate and/or nitrite can be utilized by bacteria as sources of protein nitrogen or can act as essential or non-essential hydrogen acceptors, the gaseous products of the latter methods of utilization being nitrogen, nitrous oxide and ammonia.

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# Biochemical Properties of Pork Muscle in Relation to Curing. Part II

SUMMARY—The stimulation in the presence of methylene blue of the enzymic production of nitric oxide from added nitrite by pork muscle minces under anaerobic conditions and the partial suppression by nitrite of the aerobic respiration of the minces suggested that the respiratory system is involved in nitrite reduction. Using mitochondrial preparations from pork skeletal muscle, it was shown that under anaerobic conditions the mitochondria can utilize nitrite with formation of nitric oxide. Ferrocytochrome c has been located as the immediate substrate involved, the mediating enzyme being cytochrome oxidase, suggesting that under anaerobic conditions nitrite can replace oxygen as the terminal point of the respiratory chain.

In the absence of a reducing agent nitrite oxidizes myoglobin *in vitro* to the met-form. The reduction of metmyoglobin by pork muscle minces was erratic and reduction by mitochondria has not been observed. Mitochondria can, however, readily reduce nitric oxide metmyoglobin and this observation suggests a possible mechanism for the formation of nitric oxide myoglobin during curing.

Observations on mature curing brines from Wiltshire bacon factories indicated that, although active in utilization of nitrite and nitrate, they do not produce nitric oxide. The tissue enzymes, therefore, appear to be the sole agents for producing nitric oxide and thus cured meat color.

## INTRODUCTION

THE CHARACTERISTIC PINK COLOR of cured meats is due to the partial conversion of the muscle pigment into the nitric oxide complex nitric oxide myoglobin. An essential pre-requisite is the availability of nitric oxide. A previous publication (Walters and Taylor, 1963) showed that the formation of nitric oxide from nitrite derived from the curing salts could be effected by enzyme systems persisting post-mortem in pig muscle tissue.

The present report is concerned with attempts to characterize the particular enzyme system involved and to elucidate the mechanism of formation of the nitric oxide-pigment complex. Much of the work has been done on preparations of mitochondria from pig skeletal muscle; details of the fractionation procedure used to obtain these preparations are also presented.

## EXPERIMENTAL METHODS

### Separation of skeletal muscle mitochondria

Tissue from the crura of the pig diaphragm, trimmed of fat and connective tissue, was minced in a small hand tissue grinder approximately 2 hr after the death of the animal. The minced tissue was homogenized in the tris-KCl medium of Chappell and Perry (1954) (3.1 ml per g mince) using a homogenizer in which the tissue was forced past a closely fitting rapidly rotating plastic plunger in a smooth glass tube (Aldridge *et al.*, 1960, modified as recommended by Webster and Smith, 1964); 15 movements with a plunger having a clearance of 0.02 in. were followed by a further 15 with another plunger of 0.01 in. clearance. All operations were conducted in an ice jacket.

The homogenate was diluted with the same medium to 9.0 ml per g mince and filtered through coarse gauze to remove gross particles. The filtrate was subjected to fractional centrifugation at 0–2°C according to the scheme outlined in Fig. 1. Succinic dehydrogenase was used as a marker enzyme for mitochondria, since it is associated almost exclusively with this portion of the cell and is very stable (de Duve *et al.*, 1955; Schneider and Hogeboom, 1956; Aldridge and Johnson, 1959).

The distribution of protein and succinic dehydrogenase activity in a typical fractionation are shown in Table 1. Specific activities of the mitochondrial fractions with respect to their protein content have in general been from 6 to 10 times those of the original homogenate.

In the light of the similar activities of the heavy and light mitochondrial fractions, a combined centrifugal fraction was used in routine preparations. Maximal freedom from substrates was insured by washing the mitochondrial pellet just before use and by mechanically removing lipids adhering to the sides of the centrifuge tubes.

The normal respiratory processes of mitochondria are accompanied by the esterification of inorganic phosphate of the environment. The maintenance of physiological

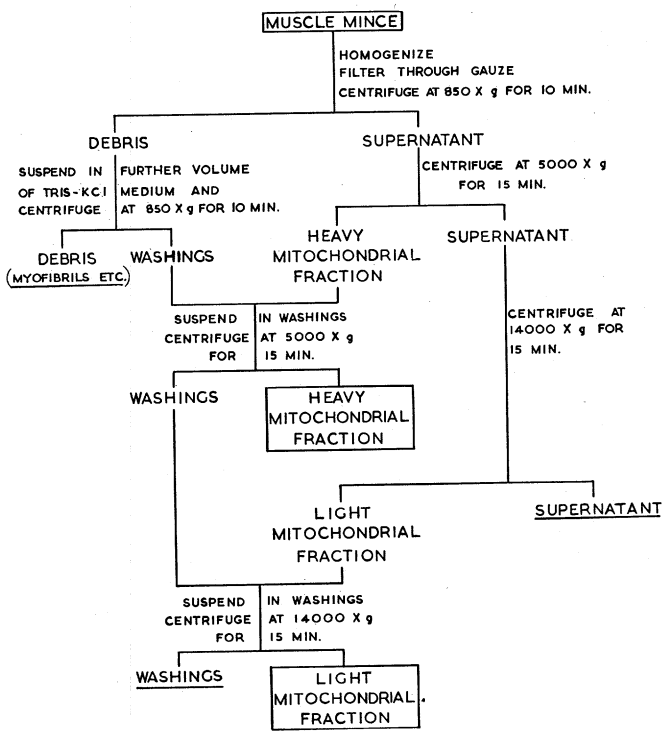


*Fig. 1. Separation of mitochondrial fractions.*

Table 1. Distribution of protein and succinic dehydrogenase activity on differential centrifugation of homogenate of pig skeletal muscle (24 g).

| Fraction | Total protein-mg | Succinic dehydrogenase activity: $\mu$l $CO_2$ per hr | | Enrichment of activity per mg protein per hr |
|---|---|---|---|---|
| | | per g original tissue | per mg protein | |
| Homogenate | 3240 | 2570 | 48 | ..... |
| Debris (up to 850 × G for 10 min) | 1580 | 2570 | 36 | × 0.75 |
| Mitochondria—heavy (up to 5000 × G for 15 min) | 92 | 1600 | 375 | × 7.9 |
| Mitochondria—light (up to 14000 × G for 15 min) | 50 | 640 | 290 | × 6.0 |
| Supernatant | 880 | 1140 | 29 | × 0.6 |
| Washings | 318 | 990 | 69 | × 1.4 |

% recovery of protein, 81%; % recovery of activity, 105%.

conditions of activity in the separated mitochondria was examined by observing the ratio of phosphate esterification to oxygen consumption in an appropriate medium. With pyruvate (11.1 m$M$) and malate (1.11 m$M$) as substrates in the oxidative phosphorylation medium of Aldridge and Parker (1960), fortified with 0.4% bovine plasma albumen (Hawtrey and Silk, 1961) an average ratio of 2.6 $\mu$moles phosphate esterified per $\mu$atom oxygen consumed was observed at an average oxygen uptake of 175 $\mu$l per mg protein per hr.

These figures compare well with the values of 2.8 and 124 respectively reported by Azzone and Carafoli (1960) for rat skeletal muscle mitochondria. Further evidence of physiological intactness was provided by an 80% reduction in oxygen uptake when the hexokinase included with glucose in the Aldridge and Parker medium to provide an ATP trapping system was omitted.

#### Mitochondrial reaction studies

Unless otherwise stated mitochondrial reactions were carried out in the Aldridge and Parker medium, modified to pH 6.0 and containing either pyruvate (10 m$M$) + fumarate (1 m$M$) or reduced nicotinamide adeninedinucleotide (10 m$M$) as substrates.

#### Nitrite utilization by mitochondria

Mitochondrial fractions containing from 0.6 to 4.6 mg of tissue protein were incubated in Thunberg tubes under argon for 60 min at 37°C with 12.5 or 25 $\mu$g of sodium nitrite. Control determinations were carried out on similar preparations inactivated before incubation by immersion for 10 min in a water bath at 80°C.

#### Nitric oxide production by mitochondria

Mitochondrial fractions containing approximately 0.3 mg of tissue protein were incubated, as described previously, with sodium nitrite (0.61 m$M$) and oxyhemoglobin (43 $\mu M$). Nitric oxide-pigment was estimated in the incubation products by dropwise addition of acetone to 80% (v/v) and measurement of optical density at 540 m$\mu$ (Hornsey, 1956).

#### Pigment reactions and differentiation

Pigment reactions were carried out in spectrophotometer cells and followed with the repetitive scanning facility of the Optica CF4R recording spectrophotometer. Anaerobic reactions were carried out in specially constructed Thunberg tube type cells and anaerobic conditions were achieved by repeated evacuation and filling of the cells with argon or nitrogen. Pigment differentiation was confirmed by using difference spectra in the Soret region, the difference in absorption between different reaction products being compared over a range of wavelengths with that between known pigment derivatives.

#### Cytochrome oxidase experiments

Aerobic oxidation of 20–25 $\mu M$ ferrocytochrome c (obtained by reduction of ferricytochrome c with a small quantity of dithionite) by the cytochrome oxidase associated with approximately 0.03 mg of mitochondrial protein was followed in the tris-KCl medium of Chappell and Perry (1954), with and without the inclusion of 50 m$M$ potassium nitrite, by rapid repetitive observation of optical density at 550 m$\mu$.

For anaerobic experiments the spectrophotometer cells contained ferricytochrome c (14–30 $\mu M$) and mitochondria (containing approximately 0.05 mg protein) with nitrite (to give 10–30 m$M$ after mixing) ± cyanide (to give 32 m$M$) in the side arm. Under anaerobic conditions, rapid and complete reduction of the ferricytochrome c was effected by preliminary incubation at 37°C prior to adding the reactants from the side arm.

#### Enzymic reduction of metmyoglobin, nitric oxide myoglobin and nitric oxide ferricytochrome c and transfer of nitric oxide from nitric oxide ferricytochrome c to metmyoglobin

Water jacketing of the spectrophotometer cells for temperature control was used in this part of the work. Nitric oxide metmyoglobin and nitric oxide ferricytochrome c were formed *in situ* from metmyoglobin or ferricytochrome c respectively by introducing gaseous nitric oxide after establishing anaerobic conditions. Excess nitric oxide was removed by further repeated evacuation and filling with argon or nitrogen.

In experiments involving the transfer of nitric oxide, scrupulous removal of nitric oxide by this method was effected before replacing the side-arm of the cell by one containing metmyoglobin and mitochondria.

Prior to tipping and incubation, anaerobic conditions were again established with the new side-arm in position. The effectiveness of the intermediate removal of excess nitric oxide was confirmed by the lack of nitric oxide metmyoglobin formation in the absence of substrate.

All incubations were conducted in Chappell and Perry (1954) tris-KCl buffer containing 20 m$M$ glucose, 4.0 m$M$ potassium phosphate and ADP (1.0 m$M$) as phosphate acceptor. Cytochrome c, metmyoglobin and substrate (reduced nicotinamide adeninedinucleotide) concentrations have ranged from 15–30 $\mu M$, 40–60 $\mu M$ and 60–400 $\mu M$ respectively. Mitochondrial additions (protein content approximately 0.1 mg) were made from the side arm after establishment of anaerobic conditions.

### Anaerobic incubation of curing brine organism inoculations

The 0.25 ml samples of brine were incubated in 50 ml of medium composed of Oxoid nutrient broth no. 2 with 5% Fildes extract, 2% defibrinated horse blood, 20% salt and 200 ppm sodium nitrite. Comparative incubations were made of minces of fresh pig quadriceps femoris muscle (2.5 g) in the same medium with the addition of chloromycetin to a concentration of 500 ppm.

## RESULTS AND DISCUSSION

### Sensitivity of Myoglobin to oxidation by Nitrite

In the nitric oxide myoglobin complex responsible for the color of cured meat the pigment is in the reduced form. Although nitrite is a necessary precursor for nitric oxide, it has an antagonistic action in that it is capable of directly oxidizing haem pigments (Colpa-Boonstra and Minnaert, 1959).

Both the oxy- form of purified pig myoglobin (Walters and Taylor, 1964a) and the oxymyoglobin contained in fresh extracts of pig skeletal muscle were very susceptible to oxidation by nitrite. At pH 6.0 and 0°C, for instance, 50 $\mu M$ oxmyoglobin was oxidized to metmyoglobin within 15 min by 2.45 m$M$ sodium nitrite (170 ppm). No spectral indication of the formation of nitric oxide metmyoglobin was observed, suggesting the absence of concurrent formation of nitric oxide.

Nitric oxide myoglobin itself was more stable to nitrite, only 7% being oxidized after 22 hr under the same conditions. It is clear that the mechanism of formation of nitric oxide myoglobin in muscle tissues must be capable of counteracting the oxidative tendency of nitrite.

### Respiratory activity and nitrite metabolism

Anaerobic gas exchange experiments with pork muscle mince (Walters and Taylor, 1963) showed marked stimulation of the production of the nitric oxide gas fraction by the inclusion of reduced methylene blue (Table 2).

No evolution of a nitric oxide gas fraction was detected on incubation of reduced methylene blue with sodium nitrite under comparable conditions in the absence of the muscle mince. Reduced methylene blue enhances the overall utilization of nitrite by pork muscle minces under anaerobic conditions. Comparative control experiments, in this case using muscle minces in which the enzyme systems had been inactivated by previous heat treatment, showed no evidence of direct chemical action between nitrite and reduced methylene blue.

Table 2. Effect of reduced methylene blue on anaerobic production of nitric oxide gas fraction by pig muscle minces at 37°C. (3 g muscle mince; 3.0 ml 0.2$M$ phosphate buffer, pH 6.0 + 0.5 ml 3.0% (w/v) sodium nitrite; chloromycetin present to 8 mg %; 0.027 mg/ml reduced methylene blue.)

| Gas evolution—$\mu$l/min | | | | | |
|---|---|---|---|---|---|
| Without methylene blue | | | With methylene blue | | |
| Alkali Absorbent | Alkaline Sulfite Absorbent | Difference (Nitric oxide gas fraction) | Alkali Absorbent | Alkaline Sulfite Absorbent | Difference (Nitric oxide gas fraction) |
| 0.72 | 0.45 | 0.27 | 1.43 | 0.71 | 0.72 |
| 0.79 | 0.39 | 0.40 | 1.24 | 0.60 | 0.64 |
| 1.16 | 0.72 | 0.44 | 1.36 | 0.63 | 0.73 |

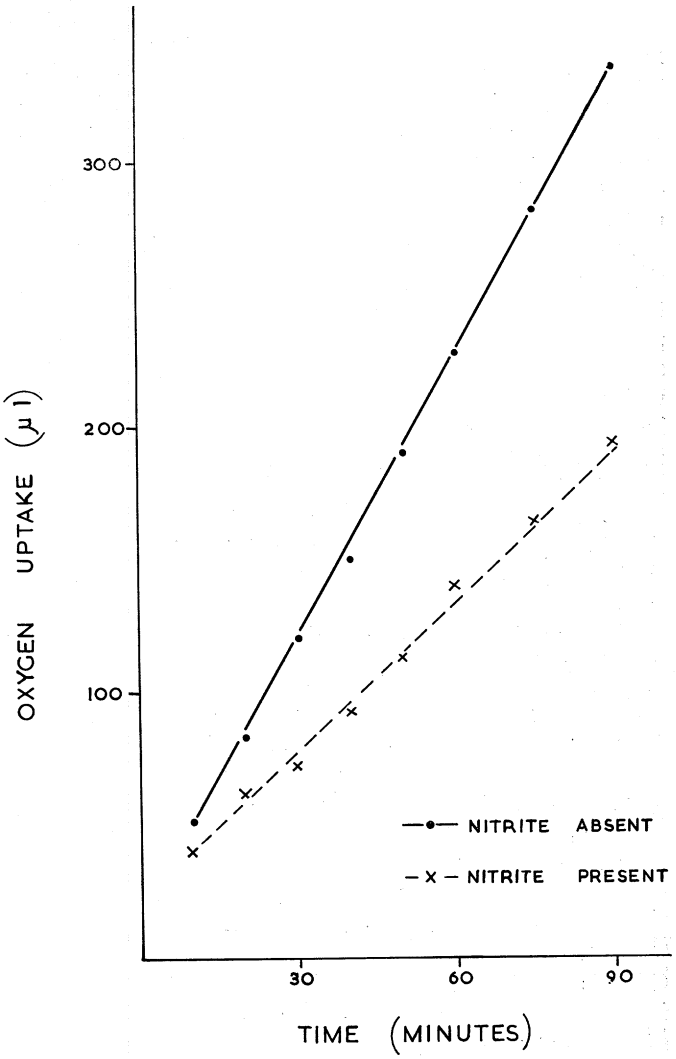


*Fig. 2. Effect of nitrite (62 mM) on aerobic respiration of pork muscle mince.*

These stimulatory effects suggest that the capacity of muscle minces to effect utilization of nitrite with the formation of a nitric oxide gas fraction is associated with the respiratory processes of the tissue. Further evidence for the implication of the respiratory systems is provided by the observations that the endogenous respiration of muscle minces in air was inhibited by nitrite (Fig. 2) and that the formation of nitric oxide myoglobin from sodium nitrite and the indigenous pigment of minces was diminished by the substitution of aerobic for anaerobic conditions (Table 3).

Table 3. Nitric oxide myoglobin formation on incubation of pig muscle minces with sodium nitrite under aerobic and anaerobic conditions at 37°C. (3 g muscle mince; 3.0 ml 0.2$M$ phosphate buffer, pH 6.0 + 0.5 ml 3.0% (w/v) sodium nitrite; chloromycetin present to 8 mg %; 90 min incubation.)

| Average nitric oxide myoglobin formed—mg[a] | |
|---|---|
| Anaerobic conditions (Atmosphere—argon) | Aerobic conditions (Atmosphere—air) |
| 2.5 | 0.8 |
| 5.2 | 2.2 |

[a] Determined by the method of Hornsey (1956).

**Mitochondria and nitrite metabolism**

Since most of the respiratory activity of the cell is located within the mitochondria, the use of preparations of these sub-cellular components suggests itself as a means of more directly implicating the respiratory mechanism. By eliminating much unwanted protein material the use of mitochondrial preparations permits direct spectrophotometric observation of pigment changes.

Preparations of mitochondria, isolated according to the procedure described in the experimental methods section, are capable of metabolizing sodium nitrite under anaerobic conditions, with pyruvate and fumarate as substrates. Nitrite utilization was negligible by controls in which the mitochondrial enzymes had been inactivated by heat treatment. The observed activities were variable, utilization ranging from 1 to 16 $\mu g$ of sodium nitrite per mg of mitochondrial protein after 1 hr at 37°C.

Formation of nitric oxide as a product of nitrite utilization by mitochondria was observed by the inclusion of oxyhemoglobin as a sensitive detector. After 1 hr at 37°C, conversion of 47–52% of this pigment to the nitric oxide form was observed by an adaptation of the Hornsey (1956) technique.

**Mechanism of formation of nitric oxide myoglobin**

*Nitric oxide ferricytochrome c as an intermediate.* The oxidation-reduction potential for the reduction of nitrite with formation of nitric oxide is +0.99 volt (Charlot *et al.*, 1958), a value in excess of that of the normal terminal aerobic reaction of the respiratory chain brought about by cytochrome oxidase:

$$O_2 + 4H^+ + 4e \rightleftharpoons 2H_2O$$

On this basis, attention was directed to the ability of the ferrocytochrome c–cytochrome oxidase complex to effect reduction of nitrite.

Fig. 3 illustrates the results of a typical experiment

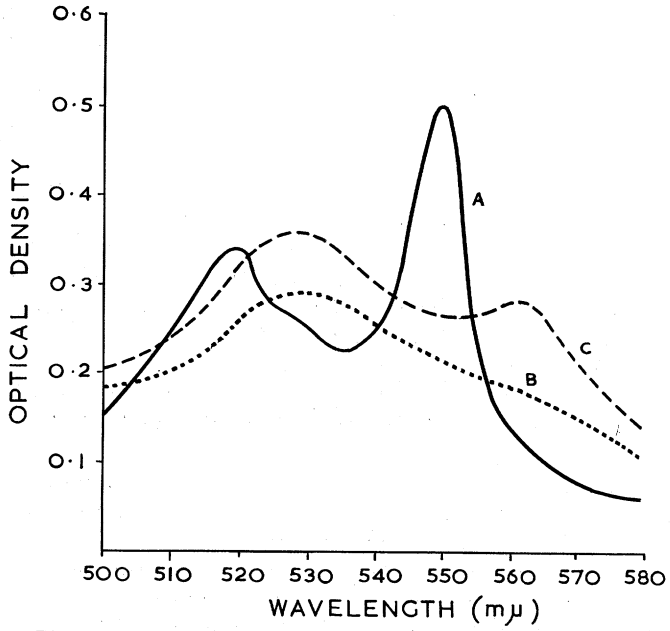


*Fig. 3. Absorption spectra of ferrocytochrome c (A) and of the products of its oxidation by ferricyanide (B) and by nitrite in the presence of mitochondria (C).*

carried out to examine this point, by following the oxidation of ferrocytochrome c during anaerobic incubation with nitrite and/or cytochrome oxidase, introduced by the inclusion of mitochondrial preparations. Curve A is the spectrum of ferrocytochrome c, as observed both initially and after overnight incubation at 37°C under anaerobic conditions with nitrite or mitochondria alone. No direct chemical oxidation of the ferrocytochrome c by nitrite was observed.

Curve B is the spectrum of ferricytochrome c, formed on addition of ferricyanide to the products of an overnight incubation containing no nitrite. Curve C shows the spectrum of the products of overnight incubation with nitrite in the presence of mitochondria. The oxidation of the ferrocytochrome c under these conditions is clearly shown by the replacement of the strong $\alpha$- and $\beta$-peaks of the ferrocytochrome c spectrum by the broad single peak of the oxidized form.

Comparison with the reference spectrum of ferricytochrome c shows a distinct additional peak at 563 m$\mu$. The spectrum of nitric oxide ferricytochrome c has a peak at this wavelength, and the appearance of this second peak confirms the partial formation of the nitric oxide derivative, thus providing a mechanism for trapping the nitric oxide resulting from the reduction of the nitrite. The ratio of the optical densities of the nitric oxide derivative and the uncomplexed ferricytochrome c at 563 m$\mu$ has been determined as about 1.9:1. On this basis the observed optical densities at this wavelength of the spectra in Fig. 3 would correspond to an approximately 85% formation of the nitric-oxide complex.

The necessity for the presence of mitochondria to effect nitrite reduction implies the responsibility of cytochrome oxidase as mediating enzyme. Confirming evidence of the participation of this enzyme we observed that the action of mitochondria in promoting oxidation of ferrocytochrome c by nitrite was completely blocked by the addition of cyanide, which is known to inhibit cytochrome oxidase action. This leads to the concept that, under anaerobic conditions, nitrite may act as a replacement for oxygen as the terminal electron acceptor of the respiratory chain, allowing continuation of respiratory activity under conditions of oxygen deprivation.

On this basis, the production of nitric oxide, with its consequent implications for color development in curing, appears as purely incidental. The affinity of cytochrome oxidase for nitrite appears, however, to be much smaller than that for oxygen. At 20°C, the rate of oxidation of ferrocytochrome c was of the order of 0.15 $\mu$mole per mg mitochondrial protein per hr, as compared with an oxygen utilization by similar preparations at 37°C averaging 7.0 $\mu$moles per mg protein per hr.

The interaction of nitrite and cytochrome oxidase provides a further example of antagonistic effects. Fig. 4 shows the effect of nitrite on the activity of cytochrome oxidase under aerobic conditions, as measured by the disappearance of the $\alpha$-peak of ferrocytochrome c. It is clear that the activity of the enzyme is suppressed in the presence of nitrite, so that in this sense nitrite tends to inhibit the activity which, by acting as terminal electron acceptor under anaerobic conditions, it simultaneously promotes.

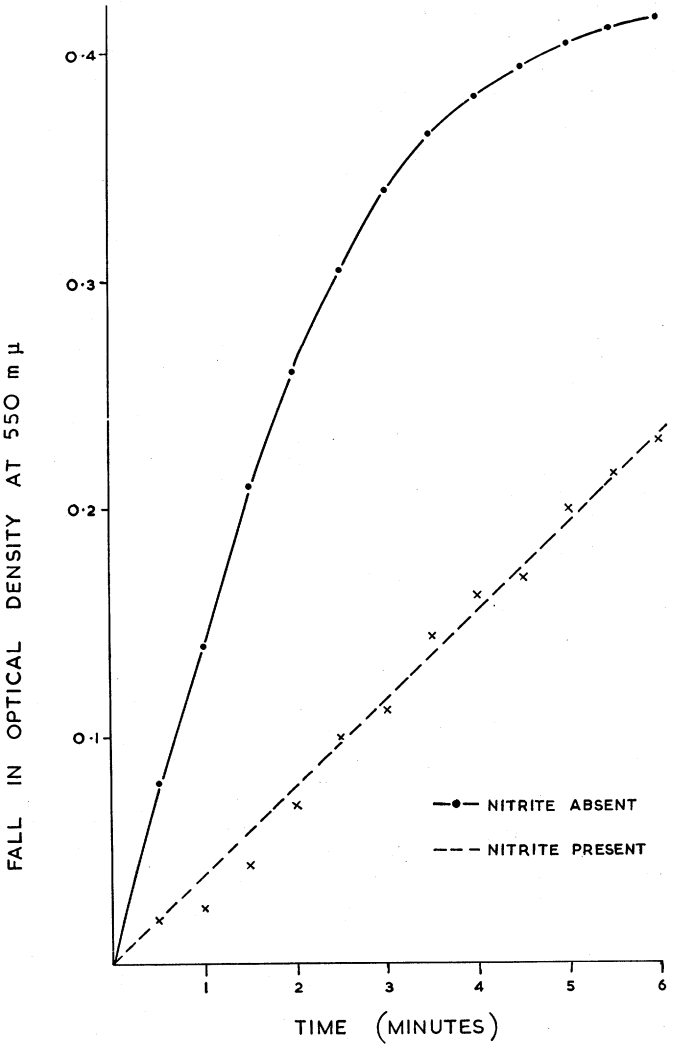


*Fig. 4. Effect of nitrite (50 mM) on aerobic cytochrome oxidase activity at 37°C.*

*Transfer of nitric oxide to myoglobin.* Ferrocytochrome c does not form a nitric-oxide derivative (Ehrenburg and Szczepkowski, 1960) so that reduction of nitric oxide ferricytochrome c must be accompanied by dissociation of the nitric oxide. This reduction can be effected by mitochondria in the presence of a suitable substrate, and the simultaneous transfer of the nitric oxide to available haem pigment can be demonstrated. The experimental evidence on this point is shown in Fig. 5. Nitric oxide ferricytochrome c and metmyoglobin were incubated anaerobically at 37°C with mitochondria, with and without reduced nicotinamide adeninedinucleotide as substrate. Fig. 5 shows the spectra initially and after incubation for 12 min. (The curves have been displaced vertically for the sake of clarity.)

Metmyoglobin was used in view of the known tendency of nitrite to oxidize the pigment to this form. The initial spectra are compounded of the spectra of nitric oxide ferricytochrome c and metmyoglobin, with a small additional peak at 550 mμ ascribed to immediate formation of a little ferrocytochrome c by the mitochondria. The final spectra show three main points of contrast in the presence and absence of the substrate: 1) in the presence of substrate a marked peak had developed at 550 mμ (the α-peak of ferrocytochrome c). 2) in the absence of substrate the spectrum showed greater optical density at 505 and 630 mμ, where the absorption of metmyoglobin is greater than that of other myoglobin derivatives. 3) in the presence of substrate the spectrum showed a very marked shoulder at 570–590 mμ in the region of the α-peaks of pig nitric oxide myoglobin (578 mμ) and nitric oxide metmyoglobin (573 mμ).

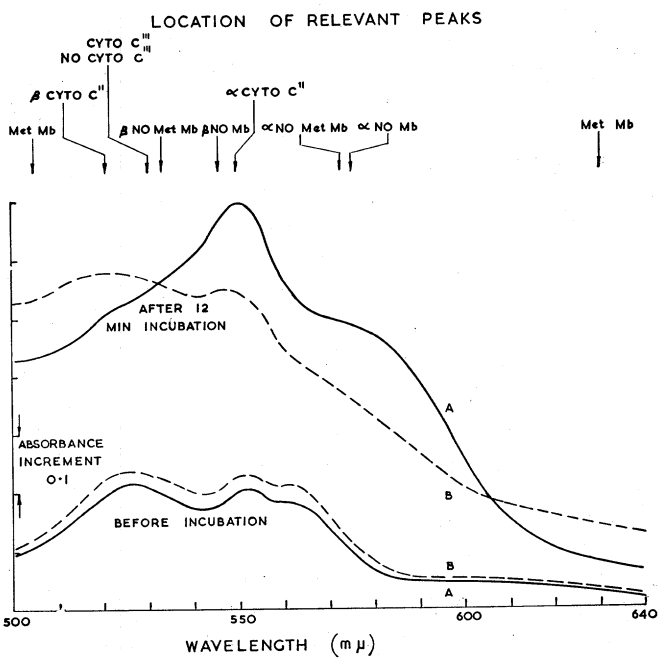


*Fig. 5. Spectra of a nitric oxide ferricytochrome c/metmyoglobin mixture before and after anaerobic incubation with mitochondria with (A) and without (B) reduced nicotinamide adeninedinucleotide as substrate.*

It is concluded that the enzymic reduction of nitric oxide ferricytochrome c has been accompanied by the formation of either nitric oxide myoglobin or nitric oxide metmyoglobin. It is possible to discriminate between these alternatives through the difference in their behavior on exposure to air. Nitric oxide myoglobin is unaffected, but nitric oxide metmyoglobin (in the absence of free nitric oxide) is converted to metmyoglobin.

Fig. 6 shows the difference spectra in the Soret region of the incubation products, after aeration and dilution, with reference to a similar dilution of a mixture initially containing ferricytochrome c in place of nitric oxide ferricytochrome c and hence retaining its original metmyoglobin unchanged. The formation of a difference spectrum shows that the pigment in the diluted products of incubation with nitric oxide ferricytochrome c was not wholly in the form of metmyoglobin. This implies that nitric oxide myoglobin, rather than nitric oxide metmyoglobin, was present in the products before dilution and aeration, and the shape of the difference spectrum itself confirms the presence of the nitric oxide derivative.

The broken curve in Fig. 6 shows the effect of chemical conversion of the residual pigment to nitric oxide myoglobin by addition of dithionite and nitrite to the diluted incubation products. The height of the difference peak was increased, but the shape of the curve was not substan-

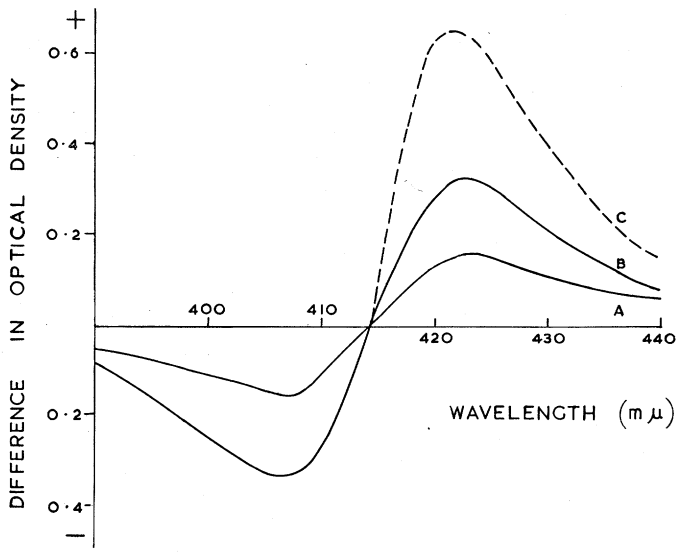


*Fig. 6. Soret difference spectra of the products of anaerobic incubation for 5 min (A) and for 40 min (B) of nitric oxide ferricytochrome c and metmyoglobin with mitochondria and reduced nicotinamide adeninedinucleotide as substrate after aeration and dilution (A and B) and after addition of dithionite and nitrite (C), with reference to similarly aerated and diluted products of a corresponding incubation containing uncomplexed ferricytochrome c in place of nitric oxide ferricytochrome c.*

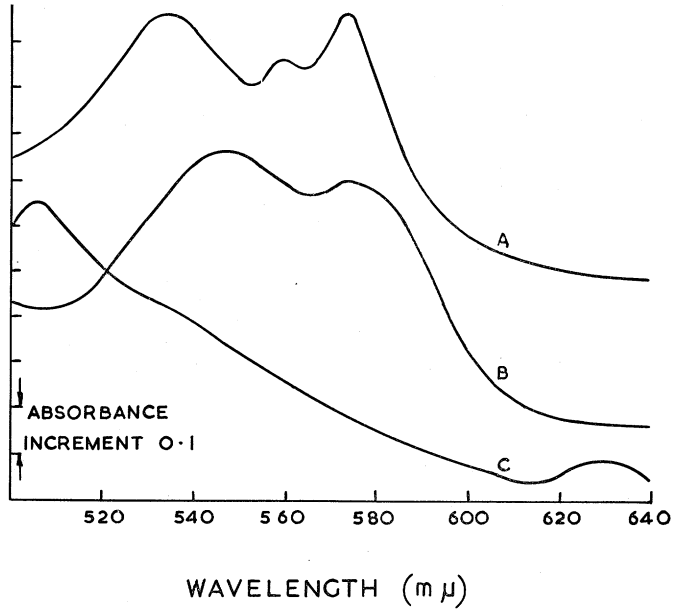


*Fig. 7. Spectra of nitric oxide metmyoglobin (A and B) and metmyoglobin (C) before (A and C) and after (B and C) anaerobic incubation with mitochondria and reduced nicotinamide adeninedinucleotide.*

tially altered. The height of the difference peak increased with increasing time of incubation, corresponding with a progressive conversion of the metmyoglobin into the reduced nitric oxide form. Moreover, the peak height after 40 minutes incubation, as assessed against the height of the peak obtained by chemical conversion, corresponded with a formation of nitric oxide myoglobin approximately equivalent stoichiometrically to the nitric oxide ferricytochrome c originally present.

Two possibilities arise: either the nitric oxide becomes associated with metmyoglobin, forming nitric oxide metmyoglobin which is subsequently reduced, or it becomes attached directly to pre-reduced pigment. The available evidence supports the first of these alternatives. Nitric oxide metmyoglobin has been shown to be reduced to nitric oxide myoglobin on anaerobic incubation with mitochondria and reduced nicotinamide adeninedinucleotide as substrate.

Fig. 7 shows the replacement of the spectrum of nitric oxide metmyoglobin (A) by that of nitric oxide myoglobin (B) after anaerobic incubation for 60 min at 37°C. Curve C shows the spectrum of metmyoglobin recorded initially and again unchanged after overnight incubation under similar conditions. The mitochondrial enzymes reduced the complexed pigment but not the uncomplexed metmyoglobin. The Soret spectra provided confirmatory evidence and permitted a rough quantitative assessment of the extent of reduction of nitric oxide metmyoglobin. After 60 min incubation at 37°C approximately 90% of the original met pigment complex had apparently been reduced.

Reduction of nitric oxide metmyoglobin in this way was not prevented by the presence of nitrite in amounts sufficient to cause rapid oxidation of oxymyoglobin. Further confirmation of the suggestion that formation of nitric oxide metmyoglobin precedes reduction of the pigment in color development is provided by earlier observations on muscle minces.

It was found that samples of mince active in converting endogenous pigment to the nitric oxide form were not consistently able to effect reduction of added metmyoglobin in the absence of nitrite. Of 60 samples of pig semimembranosus, biceps femoris and quadriceps femoris muscle, all shown to be capable of effecting conversion of indigenous pigment to nitric oxide myoglobin on anaerobic incubation with nitrite, 42 were apparently not able to reduce added metmyoglobin under anaerobic conditions. The formation of nitric oxide metmyoglobin as an intermediate has also been demonstrated by Fox and Thomson (1963) during the formation of nitric oxide myoglobin by ascorbic acid.

#### Suggested mechanism of formation of nitric oxide myoglobin during curing

A suggested mechanism for the formation of nitric oxide myoglobin during curing, based on the evidence in this report, is shown in Fig. 8. Cytochrome oxidase is widely distributed in tissues, is stable and requires no labile co-enzyme; availability of this enzyme is unlikely to be a limiting factor and, in any event, production of nitric oxide has always been observed to occur in excess. The reduction of nitric oxide metmyoglobin is readily effected by mitochondria and, in fact, proceeds spontaneously, although at a slower rate (*cf.* Keilin and Hartree, 1937). The rate limiting factor in nitric oxide myoglobin formation seems likely to be the transfer of the nitric oxide from nitric oxide ferricytochrome c to metmyoglobin, which has been shown to be substrate dependent.

#### Inability of curing brine organisms to effect nitric oxide pigment formation

Bacon curing brines are known to contain organisms able to metabolize both nitrite and nitrate and the possi-